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13. ABSTRACT

The Army requires high performance optical devices to meet diverse requirements in such areas as fire control, target recognition, laser eye protection and communications. Present devices are limited by the availability of photonic materials. To meet this need for photonic materials that have superior photochromic, photoelectric, or nonlinear optical (NLO) characteristics, we have been developing a new family of materials based on the protein bacteriorhodopsin (BR). This is a unique biological material which has promising photonic properties needed for diverse device applications such as: optical holographic memory (1), artificial photoreceptors (2), optical switches and frequency doublers (3). Genetic methods are used to produce these new BR based materials. The major objectives of this program are to gain insight as to how amino acid substitution affects photonic properties and to use these insights to identify which BR mutants have the most useful photonic properties.

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Final Report

A. Statment of Problem

The Army requires high performance optical devices to meet diverse requirements in such areas as fire control, target recognition, laser eye protection and communications. Present devices are limited by the availability of photonic materials. To meet this need for photonic materials that have superior photochromic, photoelectric, or nonlinear optical (NLO) characteristics, we have been developing a new family of materials based on the protein bacteriorhodopsin (BR). This is a unique biological material which has promising photonic properties needed for diverse device applications such as: optical holographic memory (1), artificial photoreceptors (2), optical switches and frequency doublers (3). Genetic methods are used to produce these new BR based materials. The major objectives of this program are to gain insight as to how amino acid substitution affects photonic properties and to use these insights to identify which BR mutants have the most useful photonic properties.

B. Summary of the Most Important Results

a. Development of materials with increased O lifetime

In this endeavor I also worked with Janos Lanyi of the University of California at Irvine and Akio Maeda of Kyoto University.

A central concern was the nature of the O intermediate in the photocycle of bacteriorhodopsin. This is important since two states, P(490nm) and Q(380nm), are accessible from the O state; these states are of great interest to holography and data storage since they are very long-lived and convertible. The large wavelength shift between O and P and P and Q is also of great value. Various schemes using these states for these purposes have appeared in the literature since these states were described by Hampp a few years ago. Nevertheless, practical utilization of their properties has not been obtained since the conversion of O --->P--->Q occurs with a very low quantum yield. The obvious first approach towards developing practical P and Q based materials for holography is to attempt to increase the lifetime of O.

Our physical understanding of the O state is perhaps the weakest of all the states of the photocycle. O is formed in the last step of the photocycle and is a red-shifted state. The recovery of the bacteriorhodopsin ground state from O requires the loss of a proton from the acceptor of the Schiff base proton,

D85 and the gain of this proton by a residue, X, which serves as the release group for the proton on the extracellular side in the next photocycle. Following a clue provided by the work of Ebrey and Balashov, we showed that X was E204. It was then logical to assume that if E204 was mutated to an amino acid that could not accept a proton--say E204Q--then the lifetime of O would be increased. This was indeed the case, and we published a paper detailing these results (Biochem. 35:4054-62, 1996).

We have also measured proton release into the medium after proton transfer from the retinal Schiff base to Asp85 in the photocycle and the C = O stretch bands of carboxylic acids in wild type bacteriorhodopsin and the E204Q and E204D mutants. In E204Q, but not in E204D, the normal proton release is absent. Consistent with this, a negative band in the Fourier transform infrared difference spectra at 1700 cm-1 in the wild type, which we now attribute to depletion of the protonated E204, is also absent in E204Q. In E204D, this band is shifted to 1714 cm-1, as expected from the higher frequency for a protonated aspartic than for a glutamic acid. Consistent with their origin from protonated carboxyls, the depletion bands in the wild type and E204D shift in D2O to 1690 and 1703 cm-1, respectively. In the protein structure, Glu204 seems to be connected to the Schiff base region by a chain of hydrogen-bonded water. As with other residues closer to the Schiff base, replacement of Glu204 with glutamine changes the O-H stretch frequency of the bound water molecule near Asp85 that undergoes hydrogen-bonding change in the photocycle. The results therefore identify Glu204 as XH, the earlier postulated residue that is the source of the released proton during the transport, and suggest that its deprotonation is triggered by the protonation of Asp85 through a network that contains water dipoles.

It was also important for us to examine the nature of the O state so that we could obtain additional clues about how to make long O state materials.

In the bacteriorhodopsin photocycle the recovery of the initial BR state from the M intermediate occurs via the N and O intermediates. The molecular events in this process include reprotonation of the Schiff base and the subsequent uptake of a proton from the cytoplasmic side, as well as reisomerization of the retinal from 13-cis to all-trans. We have studied the kinetics of the intermediates and the proton uptake. At moderately low pH little of the N state accumulates, and the O state dominates in the reactions that lead from M to BR. The proton uptake lags behind the formation of O, suggesting the sequence N(0)<==>O(0) + H+ (from the bulk)-->O(+1)-->BR+H+ (to the bulk), where the superscripts indicate the net protonation state of the protein relative to BR. Together with a parallel study of ours at moderately high pH, these results suggest that the sequence of proton uptake and retinal reisomerization depends on pH: at low pH the isomerization occurs first and O accumulates, but at high pH the isomerization is delayed and therefore N

accumulates. Although this model contains too many rate constants for rigorous testing, we find that it will generate most of the characteristic pH-dependent kinetic features of the photocycle with few assumptions other than pH dependency for protonation at the proton release and uptake steps

Another approach towards understanding the nature of the O state was to use diffraction determine the structure of the preceeding state N.

X-ray diffraction was used to determine the sturcture of the N intermediate. We used a mutant in which large amounts of N accumulate. The difference Fourier map revealed a major change near helix F. However the structure of N is essentially the same as that of the preceeding state M, despite the differences in the protonation state of the Schiff base. The observed structural chage near helix F will increase access of the Schiff base and Asp-96 to the cytoplasmic surface and facilitate the proton transer events that begin with the decay of M.

Using both our identification of the proton leaving group as E204 and our increased understanding of O structure and kinetics we made several bacteriorhodopsin mutants in which the O lifetime was considerably increased. This rational synthesis has been successful and we have been able to increase O lifetime from msec to 10's of seconds.

b. Development of materials with increased M lifetime

These results were reported in our previous Progress Report and are only briefly presented here.

Previous work, by other investigators, had shown that removal of the proton donor Asp 96 (D96) -> Asn 96 (D96N) greatly increases the lifetime of M. Through a close collaboration with J. Lanyi at UC Irvine we explored in more detail the mechanism by which the Schiff base is reprotonated from the cytoplasmic surface. It was determined that Thr 46 and Asp 96 together mediate proton transfer from the cytoplasmic surface to the Schiff base. One mutant, T46V, was found that accelerated the deprotonation of asp-96 while hindering or slowing proton uptake from the cytoplasmic surface. We proposed that the T46V substitution would retard M decay when it is a second mutation to D96N, even though it accelerates M decay as a single mutation. This was indeed the case, and the M decay is about 6X slower in T46V/D96N than in D96N. To our knowledge this is the first time that a nontrivial property of a BR mutant has been successfully predicted.

We have made other mutants of bacteriorhodopsin in which the yellow color is stable for more than three days. These films are used to record

holograms in real time since they have high resolution (i.e., more than 5000 lines/mm) and develop instantly (i.e.,less than 1 msec). In collaboration with MetroLaser (Irvine, CA) we have used these novel materials to:

1. Produced thick films with BR.
2. Record and reconstruct high quality holographic images in a long lifetime, erasable material (lifetime of over 3 days).
3. Made the firest demonstration of reflection mode, wavelength mulitplexed data storage in a volumetric bacteriorhodopsin material.
4. Made the first demonstration of bacteriorhodopsin base real-time holographic interferometer.
5. Demonstrated the concept of continuous, realtime double pulsed holographic interferometry using BR.

c. Investigation of the suitability of halorhodopsin as a photonic material.

Halorhodopsin is an inwardly directed chloride pump whose structure is highly homologous to that of bacteriorhodopsin. Indeed we have been able to convert bacteriorhodopsin to a chloride pump with a single amino acid substitution. It was therefore of interest to explore its properties as a possible alternative photonic material.

The light-driven chloride pump, halorhodopsin. is a mixture containing all-trans and 13-cis retinal chromophores under both light and dark-adapted conditions and can exist in chloride-free and chloride-binding forms. We resolved the multiple photochemical reactions by determining flash-induced difference spectra and photocycle kinetics in halorhodopsin-containing membranes prepared from Halobacterium salinarium, with light- and dark-adapted samples at various chloride concentrations. Careful examination of the flash-induced changes at selected wavelengths allowed separating the spectral changes into components and assigning them to the individual photocycles. According to our results, a substantial revision of the photocycle model for H. salinarium halorhodopsin, and its dependence on chloride, is required. Unlike in the earlier models, no step in this photocycle was noticeably affected when the chloride concentration was varied between 20 mM and 2 M in an attempt to identify a chloride-binding reaction.

In *N. pharaonis* halorhodopsin the chloride binding site also binds azide. When azide is bound the Schiff base deprotonates after light absorption. This azide dependent photocycle results in active proton transport in the cytoplasmic to extracellular direction. We conclude that azide fulfills the same role in this halorhodopsin as Asp-85 does in bacteriorhodopsin.

Although halorhodopsin is normally a chloride pump, it seems to have all the structural requirements--except for an internal proton acceptor and donor--of a proton pump. This result complements our earlier finding that the replacement of Asp-85 in bacteriorhodopsin with Thr converts bacteriorhodopsin to an inwardly directed proton pump.

N. pharaonis halorhodopsin contains a red shifted intermediate that may be useful for photonic applications. We are now exploring the properties of mutant halorhodopsin.

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